Proton Coupled Electron Transfer as Explored by the Tryptophan Cation Radical Formation in Biomimetic Peptides

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
B.S. in Biochemistry in the
School of Chemistry and Biochemistry

Georgia Institute of Technology
May 2014

Proton Coupled Electron Transfer as Explored by the Tryptophan Cation Radical Formation in Biomimetic Peptides

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NOMENCLATURE

PCET	Proton Coupled Electron Transfer
OEC	Oxygen Evolving Complex
EPR	Electron Paramagnetic Resonance
NMR	Nuclear Magnetic Resonance
CcP	Cytochrome c Peroxidase
CD	Circular Dichroism
SWV	Square Wave Voltammetry
DPV	Differential Pulse Voltammetry

ABSTRACT

Proton coupled electron transfer (PCET) is a mechanism exploited by many biological processes including the oxygen evolving complex (OEC) of photosystem II. PCET functions largely by the formation of a stable radical species. One amino acid with the ability to form such a stable cation radical is tryptophan. In this study, biomimetic beta-hairpin peptides have been synthesized with a cross-strand interacting tryptohan residue. The peptides have been characterized by circular dichroism spectroscopy, to be further explored by electrochemistry and electron paramagnetic resonance (EPR) spectroscopy. These techniques will provide insight into the formation of the tryptophan radical, the factors influencing its stabilization, the effect of the local amino acid environment, and, ultimately, the fundamentals of proton coupled electron transfer.

CHAPTER 1

INTRODUCTION

Proton coupled electron transfer (PCET) is a mechanism of many redox reactions by which a proton and an electron are transferred in a concerted manner. Many biological processes, such as those performed by the oxygen evolving complex of photosystem II in the chloroplasts of plants and those of the ribonucleotide reductase enzyme of DNA synthesis and repair, function by such a mechanism. An important process of the PCET reaction is the formation of a cation radical species from an amino acid residue. One such amino acid with the ability to facilitate the reaction is tryptophan, which contains an aromatic, hydrophobic side chain. The cation formation from tryptophan is also an essential reaction for the enzymatic activity of cytochrome c peroxidase (CcP), an enzyme responsible for the catalysis of H_2O_2 oxidation to form water. In an attempt to better understand the ability of and mechanism by which the cation radical forms, and ultimately by which the PCET reaction occurs, the activity of CcP and similar tryptophan containing biomolecules can be monitored.

Miller et al.[1] have effectively used site-directed mutagenesis to probe the protein environment that stabilizes the radical of compound I (or compound ES) of CcP. In the mutagenesis, the tryptophan in position 191 of the sequence was replaced by glycine or glutamine. The mutants were then crystallized and a monovalent cation binding site was observed in the cavity that was formerly occupied by the side chain of Trp-191. It was further determined that the cation binding site was created by partial negative charges in the cavity and that the Trp-191 side chain resides in a consensus potassium binding site. In a study by Huyett et al.[2], compound I of the CcP enzyme was characterized by

continuous wave and pulsed Q- band electron nuclear double resonance (ENDOR) spectroscopy. The results allowed for the complete characterization of the active site of compound I, which was found to contain an oxyferryl heme coupled to the Trp-191 π-cation radical by weak spin exchange.

In order to develop a more detailed understanding of the mechanism by which compound I forms in heme peroxidases, Hiner et al.[3] provided a focused review stating that the intermediate stores two oxidizing equivalents from hydrogen peroxide; one equivalent stored as an oxyferryl iron center and the second as a radical on the porphyrin ring or a tryptophan residue. The mechanism of action for the heterolytic cleavage of the hydrogen peroxide has been attributed to the acid-base catalytic activity of the conserved distal histidine, with assistance from the distal arginine and asparagine. Though comprehensive in its methods, results, and discussion, the review outlines an important need for more research to further understand and define the mechanism, stabilization, and environment of the cation radical formation observed in cytochrome c peroxidase. Further investigation into this process will provide valuable insight into the complex mechanism of PCET.

To propel the study of PCET by an exploration of the tryptophan cation radical formation, two water-soluble biomimetic peptides, Peptide M (IMDRYRVRNGDRIWIRLR) and Peptide W (IMDRWRVRNGDRIHIRLR) have been synthesized. These peptides were engineered to retain a hydrogen bonded beta-hairpin structure allowing for a tryptophan residue to interact with a cross-strand amino acid. The tryptophan residue interacts with the cross-strand residues of Tyrosine and Histidine in Peptides M and W, respectively. Through a series of techniques, including circular

dichroism (CD), differential pulse voltammetry, and electron paramagnetic resonance (EPR), knowledge can be gained about the formation of a tryptophan cation radical, the factors influencing its stabilization, and the effect of the local amino acid environment. Once a comprehensive characterization of the radical activity in these prototypes is completed, details of the previously unknown fundamentals of proton coupled electron transfer can be resolved and understood.

CHAPTER 2

LITERATURE REVIEW

Proton Coupled Electron Transfer

While the mechanism of PCET is not completely understood, it is known that the reaction is facilitated by the formation of a cation radical species from an amino acid residue. Tryptophan is one such amino acid whose cation radical formation is being studied. For example, Sjodin et al.[4] explored the coupling of electron and proton transfer by investigating model complexes containing an oxidized tyrosine or tryptophan residue. Through differential pulse voltammetry and laser flash photolysis with transient absorption detection of Ru-Tyr and Ru-Trp, the group was able to determine that PCET proceeds in a competition between a stepwise reaction and a single step reaction. During the stepwise process, electron transfer is followed by protonation of an amino acid radical (ETPT). In a concerted reaction (CEP), the electron and proton are transferred simultaneously. The group studied the effects of solution pH, the strength of the Ru^{III} oxidant, and the identity of the amino acid on the mechanism that would dominate the reaction. Furthermore, Zhang et al.[5] established that tryptophan oxidation is an appropriate model for understanding how electrons and protons are coupled in PCET. The team employs laser flash-quench methods to determine the intramolecular oxidation kinetics of tryptophan derivatives with water as a proton acceptor. Ultimately, the group concludes that the CEP reaction is a fundamental process that is essential for the understanding of PCET.

While the details comprising the order of electron and proton transfer are becoming clear, there is still a lack of understanding of the mechanism by which this

transfer occurs. Even then, if the mechanism is well understood and accepted, the application of the mechanism provides another obstacle to overcome. By generating alternative model structures and peptides for PCET, it could be possible to better understand the mechanism of the transfer through manipulation of amino acid residues. Furthermore, by conducting experimentation on a model structure, as opposed to the actual complex *in vitro*, the conditions of stable radical formation and PCET can be optimized and the effects of pH and the neighboring residues on the amino acid environment can be explored.

Cytochrome C Peroxidase

Knowing the role of tryptophan oxidation in the study of proton and electron coupling, the tryptophan radical and its formation should also be considered. Cytochrome c peroxidase (CcP) is an enzyme whose mechanism exploits the tryptophan cation radical. This enzyme is responsible for the catalysis of H_2O_2 oxidation to form water. In the catalytic cycle, the proximal tryptophan is known to be oxidized to a cation radical. Bonagura et al.[6] used site-directed mutagenesis and electron paramagnetic resonance (EPR) spectroscopy to introduce an ascorbate peroxidase cation binding site into CcP and show that the cation-containing mutants of CcP are no longer able to form a stable tryptophan radical. The results of this experimentation led to the conclusion that the reactivity of a redox active amino acid side chain is largely controlled by long-range electrostatic effects. Moreover, the results indicated that the oxidation and reduction of the proximal tryptophan residue is essential during the oxidation of ferrocytochrome c.

Similarly, Miller et al.[1] employed site-directed mutagenesis to probe the protein environment that stabilizes the radical of compound I (or compound ES) of CcP. The

tryptophan in position 191 (Trp-191) was replaced by a glycine or glutamine residue and these mutant forms were crystallized. The crystallization revealed a monovalent cation binding site in the cavity, previously occupied by a Trp-191 side chain. It was further determined that the cation binding site was created by partial negative charges in the cavity and the Trp-191 side chain resides in a consensus potassium binding site created by partial negative charges at the backbone of carbonyl oxygen atoms of residues at positions 175 and 177, carbonyl ends of a long alpha-helix, heme propionates, and the carboxylate side chain of Asp-235. Thus, the negative potential enveloping the side chain of the Trp-191 is sufficient to account for the stability of the radical. As more knowledge is gained about the stability of the radical, it is possible to optimize the conditions under which the tryptophan radical can be formed.

To better understand the formation of the intermediate compound I of the CcP enzyme, Huyett et al.[2] characterized the enzyme by continuous wave and pulsed Q-band electron nuclear double resonance (ENDOR) spectroscopy. The active site of compound I was found to contain an oxyferral heme coupled to the Trp-191 π-cation radical by weak spin exchange. In congruence with the results from this study, Hiner et al.[3] provided a review in which the group outlines the methods by which the intermediate stores the two oxidizing equivalents from hydrogen peroxide. One equivalent is stored as an oxyferryl iron center and the second as a radical on the porphyrin ring or a tryptophan residue. The conserved distal histidine, with assistance from the distal arginine and asparagine, is known to perform the acid-base catalysis necessary for the heterolytic cleavage of the hydrogen peroxide.

Biomimetic Peptides

With a basic understanding of the radical formation in the OEC as a foundation, biomimetic beta-hairpin peptides are being synthesized and characterized with the goal of modeling the radical formation and PCET within these proteins. Sibert et al.[7] designed a novel amino acid sequence containing a tyrosine residue. This residue can be oxidized by ultraviolet photolysis or electrochemical methods. The group characterizes the peptide with NMR spectroscopy, EPR spectroscopy, and electrochemistry to determine that the tyrosine residue couples with histidine in an interstrand α-cation interaction responsible for stabilizing the tyrosyl radical.

While peptides have been used as models for the PCET mechanism, there have yet to be studies that incorporate the cation radical formation ability of tryptophan into the peptides for the understanding of the PCET reactions of photosystem II. Based on a basic understanding of PCET and cation radical formation in the oxygen-evolving complex and cytochrome c peroxidase, biomimetic peptides have been designed to contain a tryptophan residue and maintain a hydrogen bonded beta-hairpin motif. Peptide M (IMDRYRVRNGDRIWIRLR) and Peptide W (IMDRWRVRNGDRIHIRLR) have been synthesized, allowing for the tryptophan residue to interact with the cross-strand residues of Tyrosine and Histidine, respectively.

Initially, the peptides will be characterized by circular dichroism spectroscopy, to provide valuable information regarding the secondary structure. A third peptide, Peptide A (IMDRYRVRNGDRIHIRLR) was designed and characterized by Sibert et al.[7] by the same CD methods employed here. The results of the CD were then confirmed with NMR spectroscopy and can now be used as a control for the identification of Peptide M and Peptide W secondary structure. Once the motif is established, other methods of

characterization can be applied. Similar to the methods employed by Sjödin et al.[4] and Sibert et al.[7], electrochemistry, specifically differential pulse voltammetry, can be used to measure the peak potential of the peptide across a range of pH environments. Finally, EPR, as used by Bonagura et al.[6] and Sibert et al.[7], can be used to study the unpaired electrons within the peptide.

Ideally, the tryptophan radical would be successfully generated and probed in these model peptides, allowing for the understanding of its stabilization and formation. With a fundamental understanding of the tryptophan radical formation in these prototypes and the dependency on pH, proximal amino acid environment, and charge, the mechanism of proton coupled electron transfer can be better understood and exploited for a new means of energy conversion.

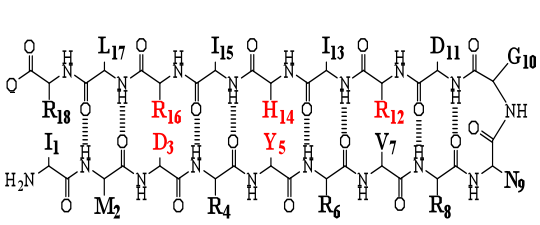
CHAPTER 3

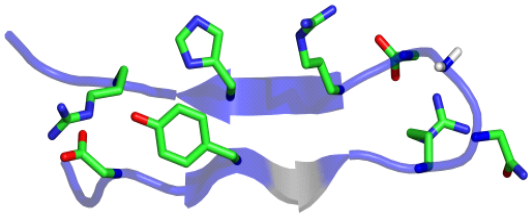
MATERIALS AND METHODS

Peptide Engineering

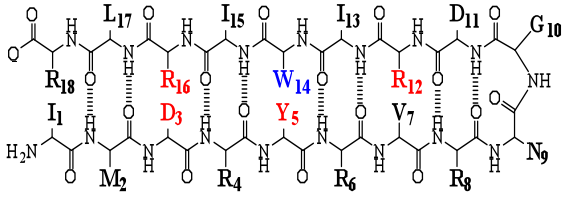
Biomimetic peptides have been engineered to contain tryptophan residues and maintain hydrogen bonded beta-hairpin motifs. Peptide M (IMDRYRVRNGDRIWIRLR) and Peptide W (IMDRWRVRNGDRIHIRLR) have been synthesized, allowing for the tryptophan residue to interact with the cross-strand residues of Tyrosine and Histidine, respectively. Peptide samples were synthesized by Genscript (> 95% purity).

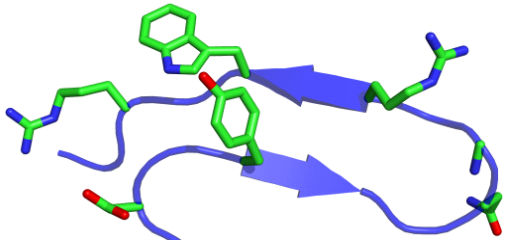
Peptide A



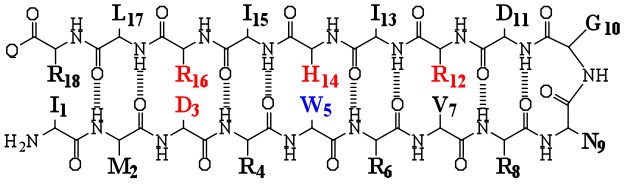


Peptide M





Peptide W



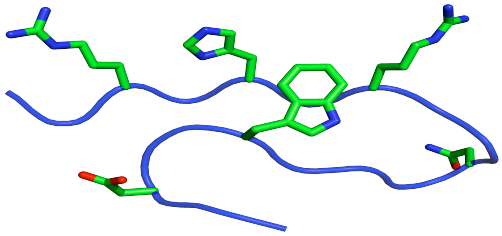


Figure 1: Beta-hairpin motifs of Peptide A, Peptide M, and Peptide W. Figures on the left show the predicted cross-strand interactions. Figures on the right show the beta-hairpin models as predicted by PEP-FOLD Structure Prediction online modeling program.

Circular Dichroism

CD spectra of 100 μM solutions were obtained using a JASCO J-810 CD Polarimeter. Spectra were collected under the following conditions: sensitivity of 100 mdeg, data pitch of 1 nm, scan speed of 50 nm/min, response time of 1 s, and a bandwidth of 1 nm. The CD spectra of peptide M and peptide W have been compared to that of Peptide A at pH 6.5. Peptide A (IMDRYRVRNGDRIHIRLR) was used as a template and is known to contain the beta-hairpin motif by the NMR spectroscopy analysis performed by Sibert et at.[7]

CHAPTER 4

RESULTS

Peptide A (Control)

Circular dichroism was employed as a method for confirming the beta hairpin secondary structure of the peptides. By passing a beam of circularly polarized light through the sample containing buffered peptide, secondary structure can be deduced by changes in ellipticity. The following spectrum was collected for Peptide A and is characteristic of a beta hairpin structure based on a minimum around 218 nm. The beta-hairpin structure for the peptide was confirmed by NMR spectroscopy, as designated in the previous literature. By using this peptide, and its CD spectrum, as a control, conclusions can be drawn regarding the secondary structure of the other peptides, Peptide M and W.

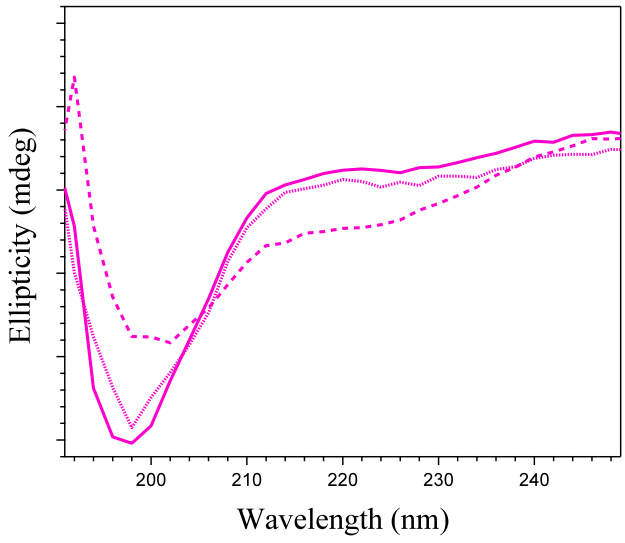


Figure 2: The CD spectrum of 100 µM Peptide A collected at a pH of 6.5 obtained at 20° C (bold line, pre-melt), 80° C (dashed-line) and at 20° C (thin-line, post-melt) showing the characteristic beta-hairpin peak. The tick mark is 1 mdeg.

Peptide M and W

Peptide M and Peptide W were studied under the same conditions. The CD data showed the characteristic minimum of ellipticity around 218 nm. Because the secondary structure was confirmed for Peptide A by NMR, comparisons that can be made between the CD spectra for Peptide A and Peptide M or W can provide valuable information regarding the secondary structure of these peptides of interest. Based on the high level of similarity, it is possible to conclude that these two peptides, M and W, share the same beta-hairpin secondary structure as Peptide A. The CD spectra collected at pH 6.5 have been provided below (Figure 3).

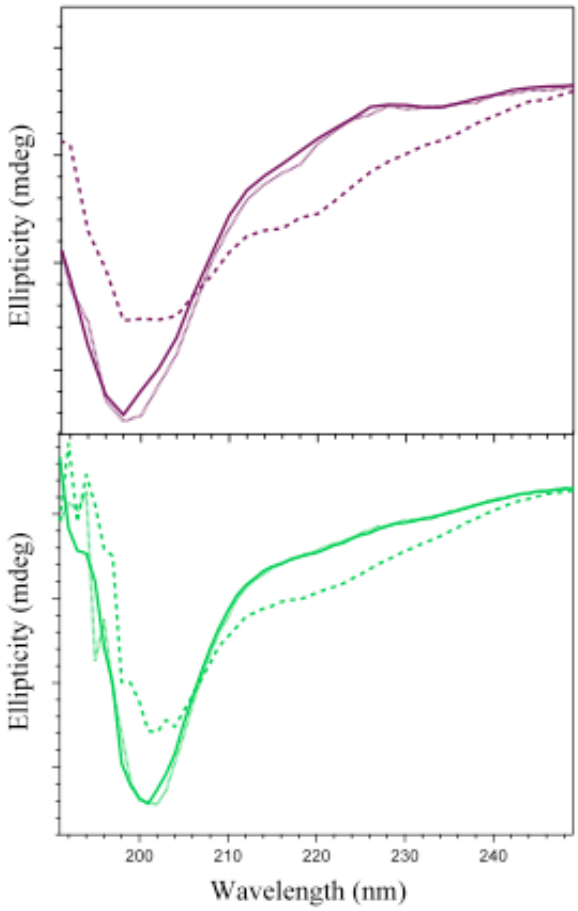


Figure 3: The CD spectrum of 100 µM Peptide M (top) and W (bottom) collected at a pH of 6.5 obtained at 20° C (bold line, pre-melt), 80° C (dashed-line) and at 20° C (thin-line, post-melt) showing the characteristic beta-hairpin peak. The tick mark is 1 mdeg.

It is also important to analyze the effects of melting on the secondary structure of the peptides. By altering the peptide environment from 20°C to 80°C, it was possible to measure the changes in ellipticity and, ultimately, determine the robustness and reversibility of the beta-hairpin folding. The CD spectra shown in Figures 2 and 3 include the measurements at 20°C before a melt, 80°C during a melt, and 20°C after a melt. The spectra taken at 80°C has a minimum ellipticity of smaller magnitude than that measured at 20°C before the melt measured at the same wavelength.

This observation is significant for two reasons. Firstly, even after melting, the peptide retains a significant amount of the beta-hairpin structure, relative to an unfolded peptide sequence. This indicates that the folding of these peptides is stable and robust, even at high temperatures. Secondly, the secondary structure that is lost to melting is reestablished upon cooling, such that the secondary structure is equivalent to that of the pre-melted conditions. The stability, rigidity, and reversibility, in congruence with the beta-hairpin conformation, make these peptides ideal candidates for further characterization.

CHAPTER 5

DISCUSSION

Based on the circular dichroism spectroscopy, it can be concluded that the peptide sequences being explored in this research, M and W, are folded into the beta-haipin motif. This conclusion was based on the analysis of the spectra in regards to both the predicted CD spectrum of a beta-sheet peptide fold, as well as the comparison to the spectrum of a peptide determined to fold into a beta-hairpin by NMR spectroscopy. Beta-hairpin motifs are expected to have a characteristic minimum of ellipticity at around 215-220 nm, which is not characteristic of either alpha helices or random coils. Both Peptide M and W spectra show this characteristic minimum, so the secondary structure of a beta-hairpin can be inferred.

To further support this conclusion, the CD spectra of these peptides were compared to the CD spectrum of Peptide A. NMR spectroscopy was employed by Sibert et al.[7] to determine the secondary structure and support the findings from the CD data for this peptide. The NMR analysis concluded that the amino acid sequence was folded into the predicted motif and supported the analysis of the CD spectrum. As such, the CD spectra of Peptide M and W can be compared to that of Peptide A, and conclusions can be drawn about the secondary structure of the peptides. Based on a high level of similarity between the three spectra, there exists sufficient evidence to conclude that Peptide M and Peptide W are folded into the beta-hairpin motif, as predicted by the PEP-FOLD Structure Prediction online modeling program.

With this conclusion, along with the high stability of the peptides, it becomes necessary and possible to further explore the peptide environment. Literature predicts that

the aromatic tryptophan residue will be crucial in a proton coupled electron transfer mechanism that can occur between the tryptophan and its cross-strand interacting amino acid residue. By applying analytical techniques, such as differential pulse voltammetry and electron paramagnetic resonance spectroscopy, to probe the electron environment, it is possible to determine the mechanism of radical formation and proton coupled electron transfer that occurs within the cross-strand interacting aromatic amino acids.

CHAPTER 6
SUMMARY

In this study, biomimetic peptides containing cross-strand interacting tryptophan residues were characterized by circular dichroism spectroscopy. By exploiting this technique in the characterization of the peptides, it was possible to determine their secondary structure. Based on the comparison of the CD spectra with that of a known beta-hairpin peptide, the secondary structure was accurately determined to be a beta-hairpin with a stable conformation. Because of the rigidity of this peptide fold, the tryptophan amino acid is held in close proximity with the tyrosine or histidine in Peptides M and W, respectively. As such, this biomimetic scaffold can be explored by other analytical techniques to develop an understanding of the cross-strand interaction. If the formation of the tryptophan radical, the factors influencing its stabilization, and the effect of the local amino acid environment can be understood, then the mechanism of proton coupled electron transfer can begin to be elucidated.

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